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Epistatic Interactions between Apolipoprotein E and Hemoglobin S Genes in Regulation of Malaria Parasitemia

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Abstract

Apolipoprotein E is a monomeric protein secreted by the liver and responsible for the transport of plasma cholesterol and triglycerides. The *APOE* gene encodes 3 isoforms Ɛ4, Ɛ3 and Ɛ2 with *APOE* Ɛ4 associated with higher plasma cholesterol levels and increased pathogenesis in several infectious diseases (HIV, HSV). Given that cholesterol is an important nutrient for malaria parasites, we examined whether *APOE* Ɛ4 was a risk factor for *Plasmodium* infection, in terms of prevalence or parasite density. A cross sectional survey was performed in 508 children aged 1 to 12 years in Gabon during the wet season. Children were screened for *Plasmodium* spp. infection, *APOE* and hemoglobin S (HbS) polymorphisms. Median parasite densities were significantly higher in *APOE* Ɛ4 children for *Plasmodium* spp. densities compared to non-*APOE* Ɛ4 children. When stratified for HbS polymorphisms, median *Plasmodium* spp. densities were significantly higher in HbAA children if they had an *APOE* Ɛ4 allele compared to those without an *APOE* Ɛ4 allele. When considering non-*APOE* Ɛ4 children, there was no quantitative reduction of *Plasmodium* spp. parasite densities for HbAS compared to HbAA phenotypes. No influence of *APOE* Ɛ4 on successful *Plasmodium* liver cell invasion was detected by multiplicity of infection. These results show that the *APOE* Ɛ4 allele is associated with higher median malaria parasite densities in children likely due to the importance of cholesterol availability to parasite growth and replication. Results suggest an epistatic interaction between *APOE* and *HbS* genes such that sickle cell trait only had an effect on parasite density in *APOE* Ɛ4 children. This suggests a linked pathway of regulation of parasite density involving expression of these genes. These findings have significance for understanding host determinants of regulation of malaria parasite density, the design of clinical trials as well as studies of co-infection with *Plasmodium* and other pathogens.

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Introduction

Children in malaria endemic areas experience multiple clinical episodes of malaria. They eventually develop acquired immunity that protects against clinical disease rather than infection. Clinical episodes occur against a background of chronic infections with multiple *Plasmodium* spp. and genotypes that can persist for months [1]. These chronic infections also represent a significant burden of disease as they contribute to anemia and can become symptomatic. Parasite density is the major determinant of whether an infection becomes symptomatic as evidenced by the fever threshold [2,3], a parasite density at which there is a >90% risk of having a malaria fever. The progression from asymptomatic infection to high parasite densities and associated clinical disease is determined by the interplay between a number of biological factors including acquired immunity, parasite virulence and host genetics.

A number of host polymorphisms have been shown to influence susceptibility to severe malarial disease [4] but there are surprisingly fewer examples of host genetics influencing *Plasmodium* blood stage infection levels *in vivo* [4,5]. Alpha-

thalassemia, haptoglobin, complement receptor 1 and glucose 6- phosphate dehydrogenase polymorphisms moderate the outcome of severe disease but do not appear to influence parasite density [4-8]. In contrast, Duffy negative erythrocytes provide the classic example of generally total refractoriness to *P. vivax* in West Africa but not to *P. falciparum* infection [9-12]. Sickle cell hemoglobin (HbS) and hemoglobin C (HbC) appear to reduce *P. falciparum* infection levels as well as modify the clinical outcome of disease [4,13,14]. While the abovementioned polymorphisms have been intensively investigated in relation to malaria, little is known about whether polymorphisms in apolipoproteins, such as human apolipoprotein E, influence susceptibility to *Plasmodium* spp. infection.

Apolipoprotein E (ApoE for protein) is a monomeric protein secreted by the liver and responsible for the binding and removal of lipids and their remnants [15]. The apolipoprotein E gene (*APOE* for gene), located at chromosome 19q13.2, encodes 3 major alleles designated *APOE* Ɛ2, *APOE* Ɛ3 and *APOE* Ɛ4, defined by two single nucleotide polymorphisms (SNPs) located in exon 4 leading to different amino acids at positions 112 (Cys for Ɛ2 and Ɛ3, Arg for Ɛ4) and 158 (Arg for Ɛ3 and Ɛ4, Cys for Ɛ2) [16]. The three isoforms encoded by these alleles have been shown to have different functional and biochemical properties [17-20] and the efficiency of these proteins is heavily determined by genotype. The most common allele in the human population is *APOE* Ɛ3 [21]. However, *APOE* Ɛ4 is thought to be the ancestral allele that has been selected against over time [21,22]. *APOE* polymorphisms have been studied in relation to several human diseases, both non-communicable and infectious. The *APOE* Ɛ4 allele has been associated with an increased risk of Alzheimer's disease, coronary heart disease and death after myocardial infarction [16,23-27] as well as several infectious diseases including human immunodeficiency virus HIV, hepatitis C and herpes simplex virus (HSV) [28,29].

The increased risk of disease in *APOE* Ɛ4 carriers is most likely due to differential blood cholesterol levels. It is well established in the cardiovascular literature that the *APOE* Ɛ4 allele is associated with elevated cholesterol compared to other alleles [15,26]. Specifically, the apoE4 proteins have been shown to be internalized and catabolized by the liver more rapidly than apoE2 and E3 isoforms, inducing a more rapid conversion of Very Low Density Lipoproteins (VLDL) to Low Density Lipoproteins (LDL) and resulting in increased cholesterol levels first in the liver and then in the plasma [19,26,30-33]. In addition, *APOE* Ɛ4 cells have also been shown to reduce fatty acid oxidation leading to accumulation of tissue and plasma lipids.

There are several potential interactions between *APOE* Ɛ4 and malaria. *Plasmodium* parasites are auxotrophic for host cholesterol [34-37], required for membrane synthesis and replication of blood and liver stages. This suggests that the increased bioavailability of cholesterol to *Plasmodium* spp. in *APOE* Ɛ4 carriers could give rise to higher parasite densities. In addition, *APOE* and *Plasmodium* sporozoites use the same receptors for cell entry leading to potential competition for binding to liver cells [38-41]. As apoE isoforms vary in strength of binding to liver cell receptors (apoE4 > E3 > E2) individuals with the *APOE* Ɛ4 allele could have less sporozoite infection in the liver [38-42] and a lower multiplicity of infection (MOI), a measure of *Plasmodium* spp. genotypes able to successfully infect and develop in the liver and succeed to patency in the blood.

To test these hypotheses, we investigated the association of *APOE* polymorphisms with *Plasmodium* spp. infection in children living in an area of seasonal malaria transmission in Southeastern Gabon. We compared *Plasmodium* spp. prevalence, density and *P. falciparum* MOI in children with different *APOE* alleles. In addition, sickle cell trait was also prevalent in this population [6] allowing us to investigate the potential interaction of this protective host erythrocyte polymorphism and *APOE* alleles in relation to *Plasmodium* spp. infection.

Results

Participant characteristics

A total of 508 asymptomatic children between the ages of 1-12 years were included in the current study investigating the association between *APOE* alleles/genotypes and *Plasmodium* spp. infection (see Table 1). 257 (50.6%) children were slide positive for any *Plasmodium* spp. with a median [IQR] *Plasmodium* spp. density of 800 [264-3060] parasites/μL. Most children were infected with *P. falciparum* (46.0%, n=234), and a few were infected with *P. malariae* (2.8%, n=14) and the prevalence of mixed *P. falciparum/ P. malariae* infection was only 1.8% (n=9). MOI was successfully genotyped for 206 of 234 *P. falciparum* positive samples and ranged from 1 to 4 (median = 2) with 112 (54.4%) infections being polyclonal (MOI > 1). Children aged 1-4 years had lower *Plasmodium* spp. prevalence (39.7%) compared to 5-9 (53.4%) and 10-12 (61.0%) year olds ($p < 0.01$). There was no significant association between age group as a categorical variable and parasite density ($p \geq 0.12$), although previously we did report an association between age and parasite density as a continuous variable [6,7]. Older children had a median MOI of 2 compared to MOI of 1 for younger children (1-4 year olds) ($p \geq 0.24$).

Host genetic analysis

APOE alleles and HbS phenotype were successfully determined in 508 and 461 children respectively (Table 1). The frequencies of the *APOE* alleles in the study population were 17.1%, 63.2% and 19.7% for *APOE* Ɛ2, Ɛ3 and Ɛ4 respectively (Table 1). For *APOE* genotypes, *APOE* Ɛ3/Ɛ3 was the most prevalent genotype in this population (43.5%), followed by Ɛ3/Ɛ4 with 111 (21.9%) and Ɛ2/Ɛ3 with 89 (17.5%) (Table 1). *APOE* Ɛ2/Ɛ2, Ɛ2/Ɛ4 and Ɛ4/Ɛ4 genotypes were relatively rare (<7%, Table 1). Of the 461 children characterized for the HbS polymorphism, 20.4% of children had sickle cell trait (HbAS). The estimated *Hb* allelic frequencies were 89.8% for the A allele and 10.2% for the S allele.

Table 1. Demographic, parasitologic and genetic characteristics of the study population.

Category	Sub-Category	N (%)
Age Groups (N=508)	1-4 years	136 (26.8)
	5-9 years	313 (61.6)
	10-12 years	59 (11.6)
Sex (N=508)	Female	235 (46.3)
	Male	273 (53.7)
***Plasmodium* spp. Prevalence (N=257)**	1-4 years	54 (39.7)
	5-9 years	167 (53.4)
	10-12 years	36 (61.0)
***Plasmodium* spp. Prevalence (N=257)**	*P. falciparum* positive	234 (46.0)
	P. malariae positive	14 (2.8)
	P. falciparum/P. malariae positive	9 (1.8)
***APOE* Alleles (N=508)**	Ɛ2	87 (17.1)
	Ɛ3	321 (63.2)
	Ɛ4	100 (19.7)
***APOE* Genotypes (N=508)**	Ɛ2/Ɛ2	27 (5.3)
	Ɛ2/Ɛ3	89 (17.5)
	Ɛ2/Ɛ4	31 (6.1)
	Ɛ3/Ɛ3	221 (43.5)
	Ɛ3/Ɛ4	111 (21.9)
	Ɛ4/Ɛ4	29 (5.7)
Hb Phenotype* (N=461)	HbAA	367 (79.6)
	HbAS	94 (20.4)

*. 461 subjects were included for Hb analysis: exclusions included subjects who could not be phenotyped for Hb (n=44) and those with small sample numbers HbSS (n=3).

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APOE and malariometric indices

The association between *APOE* alleles and malariometric indices was investigated. There were no significant associations between *APOE* alleles and the prevalence of either *Plasmodium* spp. ($p > 0.24$, Table 2), or *P. falciparum* ($p > 0.28$, Table 3) infection. There was also no association between *APOE* Ɛ3 or *APOE* Ɛ2 alleles with *Plasmodium* spp. ($p > 0.25$, Table 2) or *P. falciparum* ($p > 0.41$, Table 3) density. In contrast, as hypothesized, median parasite densities were significantly higher in *APOE* Ɛ4 children, compared to children who were non-*APOE* Ɛ4, for both total *Plasmodium* spp. (1280 vs. 640 parasite s/μL respectively, $p = 0.04$, Table 2) and *P. falciparum* (1373 vs. 631 parasite s/μL respectively, $p = 0.02$, Table 3). Interestingly, higher *P. malariae* parasite densities were also observed in children who were *APOE* Ɛ4 compared to non-*APOE* Ɛ4 (759 vs. 180 parasite s/μL respectively, $p = 0.07$). No significant associations were found with prevalence of MOI > 1 or median MOI with any of the *APOE* alleles (Table 4, $p > 0.50$) and HbAA or HbAS phenotypes ($p > 0.82$). When *APOE* alleles were further analyzed as genotypes, there was no significant difference in prevalence and density of either *Plasmodium* spp. or *P. falciparum* associated with any genotype although there was a trend of higher parasite densities in children having a genotype with one or more *APOE* Ɛ4 allele (data not shown).

Interactions between the APOE Ɛ4 allele and HbAS

Overall, there was no association between HbAS phenotype and parasite prevalence ($p > 0.46$), but HbAA children had higher *Plasmodium* spp. densities compared to children who were HbAS (800 parasite s/μL vs. 480 parasite s/μL respectively, $p = 0.05$). To analyze whether HbAS could be an effect modifier on the observed associations between the *APOE* Ɛ4 allele and parasite densities, data were stratified for Hb phenotypes (Tables 5 and 6). Analysis of parasite density distributions revealed that children who were HbAA/*APOE* Ɛ4, had significantly higher *Plasmodium* spp. and *P. falciparum* densities compared to children who were HbAA/non-*APOE* Ɛ4 (Table 5, $p = 0.01$ and Table 6, $p = 0.01$ respectively). It was also observed that children who were HbAA/*APOE* Ɛ4 had significantly higher *Plasmodium* spp. and *P. falciparum* densities compared to children who were HbAS/*APOE* Ɛ4 (Table 5, $p = 0.02$ and Table 6, $p = 0.05$ respectively). Surprisingly, no significant difference in parasite density for both *Plasmodium* spp. and *P. falciparum* were observed for children who were non-*APOE* Ɛ4 and HbAS compared to those who were non-*APOE* Ɛ4 and HbAA (Table 5, $p = 0.52$ and Table 6, $p = 0.50$ respectively) i.e. there was no additive effect of HbAS on reducing parasite density in the absence of an *APOE* Ɛ4 allele.

Table 2. The asexual malaria parasite prevalence (n (%)) and median density (value/μL, Inter Quartile Range [IQR]) in relation to the *APOE* alleles for children positive for *Plasmodium* spp. (includes *P. falciparum*, *P. malariae, and mixed P. falciparum/P. malariae*) (N=257).

	***Plasmodium* spp. (N=257)**	
***APOE* Allele Groupings (N=508)**	**Prevalence (n/N (%))**	**Density (value/μL) median [IQR]**
***APOE* Ɛ4 (N=171)**	89 (52.1)	1280 [341-3680]
non-*APOE* Ɛ4 (N=337)	168 (49.9)	640 [204-2224]
P-value	0.64	0.04**
***APOE* Ɛ3 (N=421)**	208 (49.4)	753 [232-3134]
non-*APOE* Ɛ3 (N=87)	49 (56.3)	800 [320-2765]
P-value	0.24	0.66
***APOE* Ɛ2 (N=147)**	79 (53.7)	640 [167-2000]
non-*APOE* Ɛ2 (N=361)	178 (49.3)	800 [320-3151]
P-value	0.37	0.25

The chi-square test was used to compare proportions and the Mann-Whitney U test was used for variation across the two groups. Significant associations are noted "** ".

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Table 3. The asexual malaria parasite prevalence (n (%)) and median density (value/μL, Inter Quartile Range [IQR]) in relation to the *APOE* alleles for children only positive for *P. falciparum* (excludes *P. malariae, and mixed P. falciparum/P. malariae*) (N=234).

	***P. falciparum* (N=234)**	
***APOE* Allele Groupings (N=508)**	**Prevalence (n/N (%))**	**Density (value/μL) median [IQR]**
***APOE* Ɛ4 (N=171)**	79 (46.2)	1373 [362-4328]
non-*APOE* Ɛ4 (N=337)	155 (46.0)	631 [214-2227]
P-value	0.80	0.02**
***APOE* Ɛ3 (N=421)**	190 (45.1)	753 [238-3291]
non-*APOE* Ɛ3 (N=87)	44 (50.6)	800 [320-3000]
P-value	0.28	0.69
***APOE* Ɛ2 (N=147)**	72 (49.0)	640 [175-2942]
non-*APOE* Ɛ2 (N=361)	162 (44.9)	800 [320-3497]
P-value	0.37	0.41

The chi-square test was used to compare proportions and the Mann-Whitney U test was used for variation across the two groups. Significant associations are noted "** ".

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Table 4. The asexual malaria parasite MOI median (value, Inter Quartile Range [IQR]) and MOI>1 prevalence (n/N (%)) in relation to the *APOE* alleles for children only positive for *P. falciparum* (excludes *P. malariae, and mixed P. falciparum/P. malariae*) (N=206).

	***P. falciparum MOI* (N=206)**	
***APOE* Allele Groupings**	**MOI median [IQR]**	**Prevalence MOI>1 (n/N (%))**
***APOE* Ɛ4 (N=72)**	2 [1-2]	40/72 (55.6)
non-*APOE* Ɛ4 (N=134)	2 [1-2]	72/134 (53.7)
P-value		0.96
***APOE* Ɛ3 (N=166)**	2 [1-2]	94/166 (56.6)
non-*APOE* Ɛ3 (N=40)	2 [1-2]	18/40 (45.0)
P-value		0.50
***APOE* Ɛ2 (N=68)**	2 [1-2]	35/68 (51.5)
non-*APOE* Ɛ2 (N=138)	2 [1-2]	77/138 (55.8)
P-value		0.59

The chi-square test was used to compare proportions and the Mann-Whitney U test was used for variation across the two groups. Significant associations are noted "** ".

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Table 5. The distribution of parasite asexual median density (value/μL, Inter Quartile Range [IQR]) is based on the presence of the *APOE* Ɛ4 allele and subdivided by the modifier phenotypes: HbAA and HbAS, for children positive for *Plasmodium* spp. (includes *P. falciparum*, *P. malariae, and mixed P. falciparum/P. malariae*) (N=238).

		***Plasmodium* spp. (N=238)**		
		***APOE* Ɛ4 (N=83)**	**non-*APOE* Ɛ4 (N=155)**	***P-value***
Hb Group Pooled	**Density (value/μL) median [IQR]**	1280 [320-3680]	640 [217-2291]	0.05**
HbAA (N=187)	**n**	63	124	
	Density (value/μL) median [IQR]	1461 [501-4480]	640 [219-3070]	0.01**
HbAS (N=51)	**n**	20	31	
	Density (value/μL) median [IQR]	375 [116-2720]	640 [214-1977]	0.70
	P-value	0.02**	0.52	

The Mann-Whitney U test was used for evaluate variation across the two groups. Significant associations are noted "** ".

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Table 6. The distribution of parasite asexual median density (value/μL, Inter Quartile Range [IQR]) is based on the presence of the *APOE* Ɛ4 allele and subdivided by the modifier phenotypes: HbAA and HbAS, for children positive only for *P. falciparum* (excludes *P. malariae, and mixed P. falciparum/P. malariae*) (N=217).

		***P. falciparum* (N=217)**		
		***APOE* Ɛ4 (N=74)**	**non-*APOE* Ɛ4 (N=143)**	***P-value***
Hb Group Pooled	**Density (value/μL) median [IQR]**	1327 [352-4520]	631 [214-2227]	0.03**
HbAA (N=169)	**n**	56	113	
	Density (value/μL) median [IQR]	1462 [485-5600]	631 [223-3062]	0.01**
HbAS (N=48)	**n**	18	30	
	Density (value/μL) median [IQR]	434 [123-3120]	611 [201-1973]	0.98
	P-value	0.05**	0.50	

The Mann-Whitney U test was used for evaluate variation across the two groups. Significant associations are noted "** ".

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Discussion

Data presented demonstrated a significant association between the *APOE* Ɛ4 allele and increased susceptibility to *Plasmodium* spp. infection in children exposed to intense seasonal malaria transmission. Indeed, substantially higher chronic *Plasmodium* spp. parasite densities (of both species) were observed in Gabonese children carrying the *APOE* Ɛ4 allele compared to those not having this allele. However, increased parasite density was not due to differential sporozoite competition for liver receptors because the number of infecting genomes (MOI) per child was not influenced by *APOE* Ɛ4 allele status. Thus, we propose that this increased level of parasite density results from greater cholesterol and fatty acid availability in hosts with an *APOE* Ɛ4 allele leading to increased membrane synthesis and parasite replication. This general nutrient availability mechanism of susceptibility to higher parasite densities would be a potential explanation of why the *APOE* Ɛ4 allele influences parasite density of all *Plasmodium* spp.

Another result of this study was the interaction between the *APOE* gene and *HbS* in relation to *Plasmodium* parasite density. Even if it is commonly observed that the children with the phenotype HbAS are characterized by lower parasite densities, this interaction would be best described as epistatic i.e. where the effects of one gene on the expression of a phenotype are modified by the presence of one or several other genes. Indeed, the *HbS* gene only had an effect of lowering parasite density in *APOE* Ɛ4 children as children who did not have an *APOE* Ɛ4 allele but were either *HbAA* or *HbAS* had similar low parasite densities. Hence, we conclude that the presence or absence of an *APOE* Ɛ4 allele had an overriding effect on parasite density. Importantly, we saw no additive effect of reducing parasite density in children who were *HbAS* and non-*APOE* Ɛ4. These data suggest the existence of complex epistatic interactions influencing a quantitative trait such as parasite density. Such interactions could also explain why *HbAS* is variably associated with lower parasite densities in field studies as the prevalence of the *APOE* Ɛ4 allele does vary among different study populations.

The observed epistatic interaction between genes involved in cholesterol and red blood cell (RBC) metabolism seemed intriguing and leads us to look for a linked pathway of regulation of parasite density influenced by both *APOE* and *HbS* to support the observation. Fairhurst and colleagues proposed that children with *HbAS* genotype have reduced infection levels because infected RBC of the *HbAS* genotype show lower expression of *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1) the major variant surface antigen and the parasite ligand mediating cytoadherence, and reduced capacity

to adhere [43]. A study by Frankland et al. provides a link between *APOE* and PfEMP-1 via cholesterol. They showed that depletion of cholesterol from RBC membrane inhibits the delivery or presentation of PfEMP-1 molecule to the RBC surface [44]. Similarly, Atorvastatin, a drug that lowers blood cholesterol decreases PfEMP-1 expression and cytoadherence to endothelial cells [45-47]. Consequently, in *APOE* Ɛ4 carriers more LDL and cholesterol is available to increase PfEMP-1 presentation to increase parasite survival and replication whereas those without *APOE* Ɛ4 will have lower PfEMP-1 presentation and lower parasite densities. Thus, we hypothesized that modulation of expression levels of PfEMP-1 provides a potential basis for an epistatic interaction between *APOE* and *HbS*. The overriding effect of *APOE* over *HbS* is most likely due to the role of cholesterol availability in expression of multiple phenotypes and not just PfEMP-1 expression.

This study was not designed to investigate the effect of *APOE* Ɛ4 polymorphisms on malaria morbidity outcomes. However, results revealed that *Plasmodium* spp. parasite densities were two to three times greater in children with the *APOE* Ɛ4 allele, which would increase the risk of both anemia and symptomatic malaria with parasite densities rising above the fever threshold [3]. Until today, only two field studies have investigated the association between *APOE* alleles with malaria outcomes [48,49]. Aucan et al. found no evidence for increased risk of severe malaria with any *APOE* allele in Gambian children, whereas Wozniak et al. showed that *APOE* Ɛ2/Ɛ2 was associated with early *P. falciparum* infection in infants in Ghana [48,49]. In addition to methodological heterogeneity, discrepancies between studies could be due to confounding from other host polymorphisms as observed in the current study. In our study, we demonstrated that specifically the *APOE* Ɛ4 allele was associated with higher median malaria parasite densities in West African children likely due to the importance of cholesterol availability to parasite growth and replication. To our knowledge, this is the first study involving a large enough sample size to investigate the association of *APOE* Ɛ4 alleles with the level of *Plasmodium* infection with consideration of confounding effects of sickle cell trait. Larger studies need to be completed in order to better explore the differential effect of *APOE* alleles and genotypes on susceptibility to clinical malarial disease stratifying for the confounding effect of *HbS* and potentially other host polymorphisms.

Methods

Study design and data collection

The study was performed in Bakoumba village, in Southeast Gabon near the Congo border. Malaria is highly endemic in this region with peaks of transmission at the end of the rainy seasons (September-December and March-June) [50]. A cross-sectional survey was conducted in May-June 2000 in 508 children 1-12 years of age. Details on the study population and data collection procedures have been published elsewhere [51]. Briefly, after obtaining informed consent from all parents, venous blood was collected in tubes containing EDTA for parasitological assessment for *Plasmodium* spp. by blood smears, HbS phenotyping and blood spots for genotyping [6,52]. For the present study, sufficient sample was available for *APOE* genotyping for 508 children. The study was reviewed and approved by the ethics committee of the International Center for Medical Research of Franceville, Gabon and New York University School of Medicine Ethical Review Board, United States of America.

Parasitological measurement

Parasite densities were counted per 500 leukocytes on Giemsa-stained thick blood smears and were recorded as the number of parasites per microliter of blood, assuming the average leukocyte count was about 8000/μL [53]. Duplicate readings were made for a random 15% of smears to ensure quality control.

Human genetic factors determination

The DNA was extracted from blood spots on filter paper using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Sickle cell trait was detected by *Hb* electrophoresis [6,52]. *APOE* genotypes have been determined as published with modifications [54]. Two microliters of genomic DNA was amplified with 10μM of the published primers (upstream = 5'-TCC AAG GAG CTG CAG GCG GCG CA-3', downstream= 5'-ACA GAA TTC GCC CCG GCC TGG TAC ACT GCC A-3') [55] along with 2.5μL of Q solution, 12.5μL of 2X Master Mix from the Qiagen Multiplex PCR Kit (Qiagen, California, USA), zero point five microliters (10U/μL) of Cfo/enzyme (Promega) and water up to 25μL. Then, 1μL of its buffer were incubated with 3.5μL water, 0.1μL 100X BSA and 5μL of PCR product at 37°C for 1 hour. Products have been loaded in MetaPhore 4% agarose gel (Lonza Rockland, Inc., Maine, USA) in 1X TBE according to manufacturer's instruction for electrophoresis.

Multiple *P. falciparum* infections

Multiplicities of infection (MOI) represents a measure of *Plasmodium* spp. genotypes able to successfully infect and develop in the liver, and succeed to patency in the blood. In this study, MOI was determined for *P. falciparum* by MSP2 (Merozoite Surface Protein 2) nested PCR using published primers by Falk et al. with modifications (first round: MSP2-F1 = 5'-GAA GGT AAT TAA AAC ATT GTC-3' and MSP2-1R = 5'-ATG TTG CTG CTC CAC AG-3'; second round: M5 = 5'-GCA TTG CCA GAA CTT GAA-3', N5 = 5'-CTG AAG AGG TAC TGG TAG A-3' and STail = 5'-GTT TCT TCT TAT AAT ATG AGT ATA AGG AGA A-3') [56]. Duplicate readings have been made of reaction products visualised on 1.5% agarose gel stained with EnVISION™ DNA Dye as Loading Buffer (Ambresco) to estimate the number of infections per sample.

Statistical analysis

Associations between human genetic polymorphisms and malariametric indices (parasite prevalence, density, multiplicity of infection) were tested using non-parametric Mann-Whitney U test for continuous variables and by Chi-Square test for

categorical variables. Statistical analyses were carried out using IBM SPSS Statistics Version 20 software.

For population genetic analysis, data were processed through Create V. 1.1. to convert the data for population genetics analyses [57]. We analyzed data with Fstat V. 2.9.3.2. software [58], updated from [59], which computes, estimates and tests the significance of various population genetic parameters. In this study, allele and genotype frequencies were estimated for *APOE* genotypes and *Hb* genotypes inferred from phenotypes.

For the analyses investigating the association between *APOE* genotypes/alleles with parasitological factors (density/prevalence), 508 asymptomatic children were included. For the analyses investigating the interaction between *APOE* alleles and Hb phenotypes together with parasitological factors (density/prevalence), 461 subjects from the cohort of 508 were included. Exclusions included subjects who could not be phenotyped for Hb (n = 44) as a result of limited blood sample collection and those with small sample numbers such as HbSS (n = 3). There were no statistical differences between the 461 children included for the *APOE* and Hb analyses and those who were not included in regards to the other variables ($p > 0.05$).

Conclusions

In summary, we have identified *APOE* Ɛ4 as a significant host genetic modifier of malaria parasite density in West African children. The most likely explanation for this association is cholesterol availability for parasite replication in the liver and blood. In addition, we observed an epistatic interaction between *APOE* and *HbS* genes in relation to regulation of malaria parasite density indicating a potential linked pathway of regulation of parasite density, possibly by modulating expression of the *P. falciparum major* variant surface antigen. These findings have significance for understanding host determinants of regulation of malaria parasite density, the design of clinical trials as well as studies of co-infection with malaria and other pathogens. Given a fitness cost to higher parasite densities, our data are consistent with the proposal that malaria may have selected against the *APOE* Ɛ4 allele [60].

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Author Contributions

Conceived and designed the experiments: FBL FJIF KPD. Performed the experiments: VR CMW FBL FJIF. Analyzed the data: VR CMV KET FBL FJIF KPD. Contributed reagents/materials/analysis tools: FBL FMN PD AJL FJIF KPD. Wrote the manuscript: VR KET FJIF KPD.

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